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# Thermodynamic analysis of the kinetics reactions of the production of FAME and FAEE using Novozyme 435 as catalyst

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ABSTRACT

Biodiesel is a biofuel expected to become a substitute for petroleum diesel. One of the most promising technologies for production of biodiesel is enzymatic catalysis. However, low catalytic performance of most of the enzymes employed makes such processes expensive and time-consuming. This work describes a kinetic study of the enzymatic production of biodiesel at different temperatures using either methanolysis or ethanolysis, using immobilized lipase from *Candida antarctica* (Novozym 435) as catalyst. Reactions kinetics were followed by GC, and data were used to perform thermodynamic analysis of the transition state using Arrhenius equation. We found that methanolysis is faster than ethanolysis at temperatures above 13 °C. Thermodynamic analysis of the kinetics of reactions showed that methanol is favored as acyl acceptor due to the positive activation entropy change of reaction. These data may be useful in the development of new enzymes and new processes for enzymatic production of biodiesel.



## 1. Introduction

Biodiesel has become an important renewable biofuel since it was demonstrated to be an alternative to petroleum-based energy. Biodiesel is considered a carbon-neutral fuel and a pivotal advantage related to the emission of greenhouse gases because the carbon present in its exhaust is removed by the plant source from the atmosphere. Biodiesel can be produced by transesterification of triacylglycerol (TAG)* usually obtained from vegetable oils or beef tallow, to short-chain alcohols forming fatty acid alkyl esters and glycerol [1,2]. Usually, biodiesel is produced by alkaline chemical catalysis with methanol as acyl acceptor, forming fatty acid methyl ester (FAME). However, longer-chain alcohols, such as ethanol, can be used, in which case fatty acid ethyl esters (FAEE) are formed [2]. Although chemical transesterification yields high levels of conversion of TAG into FAME with short reaction times, recovery of glycerol and removal of catalyst constitute energy consuming drawbacks. Further, the alkaline or acid waste water that is produced requires further treatment. Also, the presence of free fatty acids (FFA) and the water in the feed stock oil interfere to the chemical reaction. Lately, many heterogeneous catalysts have been tested in transesterification reactions, including immobilized lipases [3]. Lipases (E.C.3.1.1.3) are enzymes with potential for use in biodiesel production since enzymatic reactions can overcome the problems mentioned above. Although lipase-catalyzed biodiesel production possesses certain advantages, the process has not yet been adopted on an industrial scale due to certain constraints like the high cost of enzyme production, exhaustion of enzyme activity, and enzyme inhibition by methanol. This inhibitory effect is attributed to the polar coating effect alcohol has on the surface of the enzyme [4,5], and many strategies have been studied in order to overcome this problem. Among these strategies, one can note the use of co-solvents, such as *t*-butanol [6–8], long-chain alcohols (butanol) as acyl acceptors [9], and the stepwise addition of methanol [10–13]. For this work, we chose *t*-butanol as co-solvent since, this system configuration is the best experimental design for such kinetics studies.

Lipases from different sources have been tested for biodiesel production by transesterification of vegetable oils, most of them using methanol. Methanol is the alcohol mainly utilized for industrial production of biodiesel due to its low cost and abundant availability [3]. However, ethanol-based production would be an economically viable alternative in ethanol-producing countries, such as USA and Brazil. Ethanol can be obtained from vegetable sources, such as corn or sugarcane, which makes the resulting FAEE-based biodiesel a fully renewable biofuel. The high cost associated with the dehydration of ethanol is one of the major problems encountered in the industrial

*Abbreviations:* FAME, fatty acid methylester; FAEE, fatty acid ethylester; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; FA, fatty acid; FFA, free fatty acid.

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production of FAEE. Enzymatic production could prove useful in overcoming this problem, since it has been demonstrated that minor water-related activities do not interfere in enzymatic reactions [5,14,15].

The thermodynamics of enzymatic production of biodiesel has not been investigated thoroughly and it is the kinetics studies of these enzymes that provide the essential parameters for the selection of the best systems for production of biodiesel [16]. In this paper, the kinetics of enzymatic production of FAME and FAEE from rapeseed oil, as a function of reaction temperature, was investigated using immobilized *Candida antarctica* lipase (Novozym 435) as catalyst. Lipases catalyze the conversion of TAG into biodiesel in at least three reversible steps with diacylglycerol (DAG) and monoacylglycerol (MAG) as intermediates. Novozym 435 has been shown to convert TAG into FAME without significant accumulation of MAG and DAG, indicating that the conversion of TAG to DAG is the rate-determining step of the reaction [17]. Assuming that the rate constant of TAG conversion to DAG reflects the rate constant of the whole reaction, we analyzed the rate of consumption of TAG during the enzymatic biodiesel production process using GC to determine the effect of temperature on enzymatic activity. For this purpose, we used either methanol or ethanol as the acyl acceptor. Changes in the rate constant of FAME and FAEE production as a function of temperature enable us determine the transition energies involved in enzymatic biodiesel production. To analyze this data, we used the Arrhenius plot method, which correlates the dependence of the rate constants of the reaction on temperature and enables estimation of the changes in enthalpy ($\Delta H^{\ddagger}$), entropy ($\Delta S^{\ddagger}$) and Gibbs free energy ($\Delta G^{\ddagger}$) of the transition state. These data form the basis necessary for studies on the improvement of the enzymatic processes using lipases to make the enzymatic industrial production of biodiesel viable.

## 2. Materials and methods

FAME and FAEE (biodiesel) were synthesized in a 50 ml round-bottom reaction flask, attached to a reflux condenser, containing 10 ml of rapeseed oil, methanol or ethanol in a 6:1 alcohol to oil molar excess, 1% (w/v) immobilized lipase from *Candida antarctica* (5000 U/g), and 20% (*v*/*v*) *t*-butanol. Using magnetic stirring to keep the system vigorously stirred the reaction temperatures were controlled between 30 and 60 °C in a silicon oil bath. The alcohol-to-oil ratio was calculated based on the average molecular mass of rapeseed oil based on its fatty acid composition [19]. At the times noted under the figures, 500 μl samples were collected and added to 500 μl of hexane in order to arrest the reaction. The samples were centrifuged at 10,000 rpm for 5 min in a spin centrifuge at 5 °C to separate the immobilized enzyme from hexane-soluble reactants and products. Gas chromatography (GC) analysis was performed using 50 μl aliquots of clear supernatant diluted in 5 ml hexane.

The GC analyses were carried out in a Varian CP-3800 gas chromatograph equipped with a 15 m × 320 μm capillary Select™ biodiesel for glycerides column. The injection was done at 100 °C. Temperature gradient sequences between 50 °C and 380 °C were as follows: 50 °C for 1 min; from 50 °C to 180 °C at 15 °C/min; from 180 °C to 230 °C at 7 °C/min, and from 230 °C to 380 °C at 30 °C/min. Detection was by FID at 380 °C. Retention times, as well as integration of TAG, DAG, and MAG peaks were determined using GC standards from Merk (Darmstadt, Germany).

The yields of FA conversion from TAG to FAME were calculated from GC peak integrations derived using Eq. (1):

$$R = \frac{(A_{\mathrm{FAME}} / \mathrm{mm}_{\mathrm{FAME}})}{(A_{\mathrm{FAME}} / \mathrm{mm}_{\mathrm{FAME}}) + (A_{\mathrm{MAG}} / \mathrm{mm}_{\mathrm{MAG}}) + 2(A_{\mathrm{DAG}} / \mathrm{mm}_{\mathrm{DAG}}) + 3(A_{\mathrm{TAG}} / \mathrm{mm}_{\mathrm{TAG}})} \quad (1)$$

where $A_{\mathrm{FAME}}$, $A_{\mathrm{MAG}}$, $A_{\mathrm{DAG}}$, and $A_{\mathrm{TAG}}$ are the integration peaks of FAME, MAG, DAG and TAG, respectively; $\mathrm{mm}_{\mathrm{FAME}}$, $\mathrm{mm}_{\mathrm{MAG}}$, $\mathrm{mm}_{\mathrm{DAG}}$, and $\mathrm{mm}_{\mathrm{TAG}}$ are the average molecular masses of FAME, MAG, DAG, and TAG, respectively, of rapeseed oil taking into account its fatty acid composition [19].

All reagents used were of analytical grade purity. Hexane, ethanol, methanol, and *t*-butanol utilized were anhydrous. In order to remove residual water present in the rapeseed oil, the sample was heated at 120 °C for 10 min before the production experiments were conducted.

The immobilized lipase (Novozym 435) used was kindly provided by Novozymes Latin America.

## 3. Results and discussion

Kinetics of the enzymatic transesterification reaction of rapeseed oil was measured using either methanol or ethanol as acyl acceptor. Enzymatic activity, carried out with Novozym 435, was evaluated by computing the reduction of area of the TAG peak in the gas chromatogram. As conversion of TAG to DAG is the rate-limiting step in enzymatic production of biodiesel [18], this step was used to determine the rate constant of the overall reaction. Control experiments have been performed in order to evaluate the reproducibility of the reaction system regarding temperature control and agitation velocity. No influence of agitation velocity on the reaction rate constant were observed if the magnetic steering was kept above ~200 rpm (data not shown), suggesting that at such steering speed the reactions` rate were not limited by mass transfer processes. Fig. 1 is a typical chromatogram of FAME obtained after 22 h of enzymatic transesterification of rapeseed oil at 60 °C. The sample was prepared

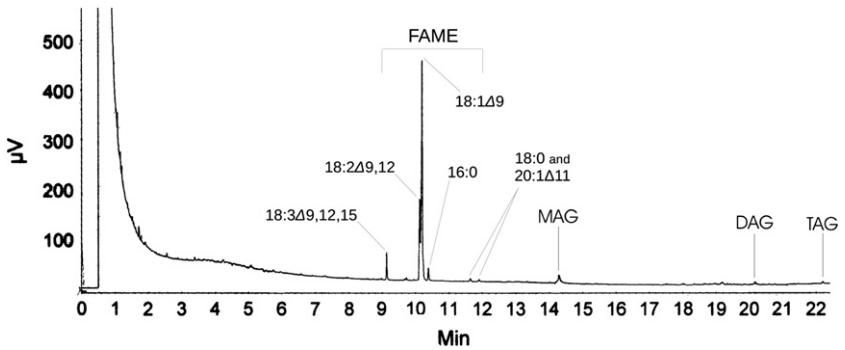


**Fig. 1.** Gas chromatogram of a typical product of enzymatic production of biodiesel. Samples from methanolysis reaction performed at 60 °C were taken after 22 h of reaction and analyzed as described in Material and methods. Peaks for FAME, MAG, DAG, and TAG are depicted. FAME peaks can be observed for the major FAs present in rapeseed oil. The composition of FAME is in accordance with the FA composition of rapeseed oil [19].

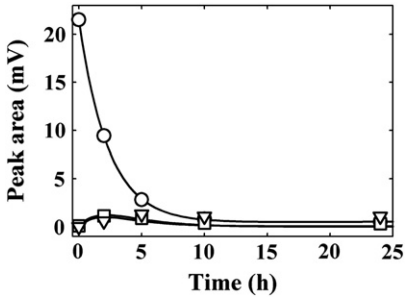


**Fig. 2.** Kinetics for integrations of TAG, DAG, and MAG peaks. Area of TAG, DAG, and MAG peaks were automatically integrated in each reaction time sample. TAG (circles) consumption follows a single-decay fashion. DAG (squares) and MAG (triangles) cannot be quantitatively measured and, in these experiments, were of concentrations too small to be precisely measured by GC.

as described in Materials and methods. The fatty acid composition of the FAME produced tallies with the fatty acid composition of rapeseed oil: 60.1% oleic acid, 21.5% linoleic acid, 9.9% $\alpha$-linolenic acid, 5.1% palmitic acid, 1.7% stearic acid, and 1.4% 11-eicosanoic acid [19]. Yields of biodiesel production were calculated as described in Materials and methods and in a typical FAME production experiment 98% of TAG was seen to have been converted into FAME at 60 °C. Such conversion is similar to the highest conversion yields reported for enzymatic biodiesel production elsewhere [3].

As described by other authors [6–8], no enzymatic FAME or FAEE production was observed in the absence of *t*-butanol. Furthermore, no conversion was observed if immobilized enzyme was not added as well (data not shown).

Fig. 2 shows the integration of the TAG, DAG, and MAG peaks as a function of the reaction time. It was observed that the diminution in the TAG peak was mono-exponential. The transesterification reaction was almost complete at 10 h (94%) and no significant accumulations of DAG and MAG were visible. DAG and MAG are not directly measurable with quantitative precision by GC [20]. However, TAG peak integration quantitatively represents the mass of TAG present in the reaction medium. The rate constant for TAG consumption can, therefore, be used to measure the rate constant of the whole reaction because it has been shown to be the limiting step for enzymatic transesterification when Novozym 435 is used [18]. In this sense, the diminution of the TAG peak in gas chromatography was used to estimate the kinetics of the transesterification reaction for enzymatic production of biodiesel from rapeseed oil.

Kinetics of the enzymatic production of FAME and FAEE were measured as a function of temperatures in the range between 30 and 60 °C (Fig. 3A and B). Rate constants of transesterification reactions fitted a single-decay equation, suggesting a first-order reaction. Darnoko and Cheryan [21] assert that alkaline (KOH)-catalyzed conversion of TAG into DAG occurs in a second order reaction, while Freedman et al. [22] found that acid-catalyzed transesterification was a pseudo-first-order reaction. Our results suggests that enzymatic conversion occurs in the first (or pseudo-first) order reaction, as is observed in acid catalysis, since all kinetic data curves fitted mono-exponential equations with a high correlation coefficient ($R$>0.99). Double-order equations were also tried to fit the data, but the regression results did not correlate so well ($R$<0.7; data not shown). The dependence of the rate constant of enzymatic biodiesel production as a function of temperature was used to calculate the transition energies for this process using the Arrhenius plot (Fig. 3C). The Arrhenius plot correlates the natural logarithm of the rate constant of the reaction [ln($k$)] with the inverse of temperature (1/$T$), in Kelvin. Transesterification performed by lipases can be considered as combination of reversible reactions of different reactants/products [16,17]. Considering the reversibility of the enzymatic transesterification and assuming that ln($k$) changes linearly with 1/$T$, it is possible to estimate the Gibbs free energy change ($\Delta G^{\ddagger}$), the enthalpy change ($\Delta H^{\ddagger}$), and the entropy change ($\Delta S^{\ddagger}$) of the transition state by use of the Gibbs equations:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \tag{2}$$

and

$$\Delta G^{\ddagger} = -RT\ln(k\hbar / k_b T) \tag{3}$$

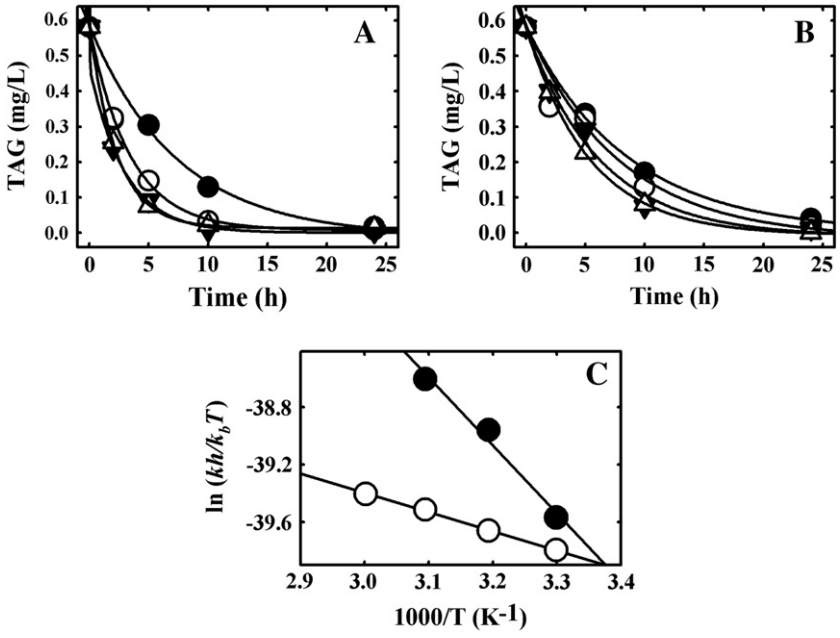


**Fig. 3.** Kinetics of enzymatic production of biodiesel. Kinetics of the enzymatic production of biodiesel was measured by the consumption of TAG at 30 °C (circles), 40 °C (triangles), 50 °C (diamonds), and 60 °C (squares), using methanol (A) or ethanol (B) as acyl acceptor. Data points were fitted with a single-order equation to calculate the kinetic rate constants of each reaction. Arrhenius analysis was done by plotting ln($k$) as a function of 1000/$T$ (C), as described in the text. Slopes and intercepts for methanolysis (closed circles) and for ethanolysis (open circles) from the Arrhenius plots were determined by linear regression.

where $k_b$ is Boltzman's constant and $\hbar$ is Planck's constant. $R$ and $T$ have their usual meanings.

Replacing $\Delta G^{\ddagger}$ in Eq. (2):

$$-RT\ \ln(k\hbar / k_b T) = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (4)$$

and rearranging the equation gives

$$ln(k\hbar / k_b T) = -\left(\Delta H^{\ddagger} / RT\right) + \left(\Delta S^{\ddagger} / R\right). \quad (5)$$

The Arrhenius plot treats Eq. (5) as a linear regression where $y = \ln(k\hbar/k_bT)$ and $x = 1/T$. In such a case, the slope gives $-\Delta H^{\ddagger}/R$, and the intercept $\Delta S^{\ddagger}/R$.

Fig. 3C shows the Arrhenius plot of enzymatic biodiesel production at temperatures of 30, 40, 50, and 60 °C. From Arrhenius plot, it is possible to confirm that $\ln(k)$ appears to change linearly with $1/T$ in the studied temperature range as expected for a single rate-limited thermally activated process [23]. When methanol is used as the acyl acceptor, the enzymatic production of biodiesel displays higher $\Delta H^{\ddagger}$ than when ethanol is the acyl acceptor. This is obvious from the higher slope of the methanolysis curve (Fig. 3C, open circles). Such information in isolation would lead to the conclusion that methanolysis transesterification presents higher activation heat than ethanolysis and might take place at a slower pace, especially at low temperatures. In fact, our calculations suggest that, if enzymatic production of biodiesel was carried out at temperatures below 13 °C, the ethanolysis reaction would be kinetically favored over methanolysis. However, in temperatures above 13 °C, methanol is kinetically favored as the acyl acceptor due to differences observed in the entropy change of the activation state ($\Delta S^{\ddagger}$), given by the intercept of curves in the Arrhenius plot (Fig. 3C). The $\Delta S^{\ddagger}$ for methanolysis appeared quite positive at 60 °C, indicating that it is entropically favorable. Fig. 4 displays the energy diagram for reagents and intermediates ($^{\ddagger}$) of enzymatic transesterification at 60 °C. The estimated values for the transition state energies are summarized in Table 1. These results suggest that when methanol is the acyl acceptor, the kinetics of biodiesel production by enzymatic transesterification of rapeseed oil is entropically favored. At high temperatures (above 13 °C), the positive $T\Delta S^{\ddagger}$ component of activation Gibbs free energy ($\Delta G^{\ddagger}$) compensates the difference between the $\Delta H^{\ddagger}$ values for methanolysis and ethanolysis. At 60 °C, $\Delta G^{\ddagger}$ estimated for methanolysis was 3.4 kJ mol$^{-1}$ lower than for ethanolysis (Fig. 4). This difference in activation free energy is responsible for the faster kinetics of the methanolysis reaction at 60 °C.

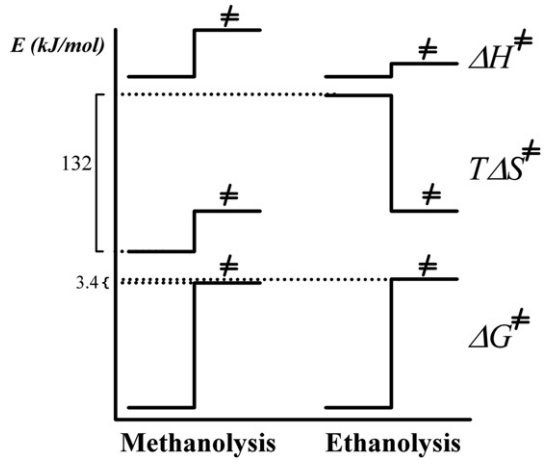


**Fig. 4.** Transition energy changes for the enzymatic production of biodiesel. Changes in the energies of the transition state ($^{\ddagger}$) for enzymatic methanolysis (left) and ethanolysis (right) were estimated from Arrhenius plots. $\Delta H^{\ddagger}$, $T\Delta S^{\ddagger}$, and $\Delta G^{\ddagger\ddagger}$ were calculated as described in the text. Energy values are shown in Table 1.

**Table 1**
Energies changes of the transition state of enzymatic production of biodiesel.

| | Acyl acceptor | |
|---|---|---|
| Energy | Methanol (kJ mol$^{-1}$) | Ethanol (kJ mol$^{-1}$) |
| $\Delta H^{\ddagger}$ | 139.5 | 11.1 |
| $T\Delta S^{\ddagger}$ | 33.9 | -98.0 |
| $\Delta G^{\ddagger}$ | 105.6 | 109 |

The difference between the standard molar entropies ($S^0$) of methanol and ethanol could account for part of the entropic differences observed. $S^0$ of methanol is 126.8 J mol$^{-1}$ K$^{-1}$ and of ethanol 160.7 J mol$^{-1}$ K$^{-1}$ [23], which account for a difference of 11.3 kJ mol$^{-1}$ at 60 °C between the methanol and ethanol $TS^0$ components. As shown in Fig. 4, if one assumes that the transition states for methanolysis and ethanolysis reactions have similar $S^{\ddagger}$ values, a difference of 132 kJ mol$^{-1}$ would be observed between the $T\Delta S^0$ values of methanol and ethanol. While we believe that $S^0$ values of methanol and ethanol could be part of the explanation, other factors might be involved in the entropy compensation of enzymatic methanolysis of biodiesel production. Intuitive conclusions point to a possible lower organization of methanolysis intermediates. Thus, in addition to the explanation based on the $S^0$ values of methanol and ethanol, our present results might be due also to differences between the bulk solution and/or in the enzyme active site organization when alcohol molecules bind to the enzyme or to the distinct conformational surfaces of lipase molecules when dissolved in methanol rather than when dissolved in ethanol.

Although enzymatic mechanisms of most lipases are well described, they may be significantly distinct for the reaction in biodiesel production for industrial purposes. Most lipases work *in vivo* in the water–micelles interface, while industrial biodiesel production is carried out in oil–alcohol monophasic mixtures, where no micellar systems are present. Inhibition of lipase activity in such media by methanol has been reported when the alcohol/oil molar rate exceeds 1. Strategies used to avoid inhibition include stepwise addition of methanol [10–13] and the use of co-solvents to reduce the superficial tension between oil and methanol [7–9]. Even when co-solvents such as *t*-butanol are used, the oil–alcohol mixture remains homogeneous.

The mechanism of the kinetics of the enzymatic production of biodiesel has not been thoroughly investigated and more studies are required to answer such questions. The mechanism of the enzymatic catalysis for biodiesel production needs to be clearly elucidated to enable the development of more specific lipases for biodiesel production. Such development may involve the selection of new classes of lipases and site-directed mutagenesis strategies in order to find, or design, active sites and protein surfaces with chemical properties more favorable for the transesterification reaction in the industrial production of biodiesel.

## 4. Conclusions

In this work, kinetic studies on the biodiesel production by enzymatic transesterification of rapeseed oil are presented using immobilized lipase from *Candida antarctica* (Novozym 435) either through methanolysis and ethanolysis. Thermodynamic analysis of the transition states of reaction in several temperature indicate that methanolysis is kinetically favored in relation to ethanolysis due to the positive activation entropy observed in methanolysis reaction. However complete explanations of lipase mechanism on the transesterification reaction demands further studies, the present results might be due to, at least one of the following: (i) partition of methanol molecules from the bulk into the lipase active site generates more molecular disorder in the bulk than by the partition of ethanol

molecules; (ii) binding of ethanol to amino acid residues present in the lipase active site must lead to a more ordered cluster of atoms than with methanol binding; and (iii) as acyl acceptor alcohol is one of the solvents present in the reaction media (16% for methanol and 22% for ethanol), the higher polarity of methanol must affect the lipase surface, making the entropy of the complex enzyme-TAG-methanol higher than with enzyme-TAG-ethanol. In any case, the differences observed in $\Delta S^{\ddagger}$ values between the methanolysis and ethanolysis reactions may be due to the differences in the bulk solution and/or in the enzyme active site organization when alcohol molecules bind to the enzyme or to the distinct conformational surfaces of lipase molecules when dissolved in methanol rather than when dissolved in ethanol.

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## References

[1] H. Fukuda, A. Kondo, H. Noda, Biodiesel production by transesterification of oils, J. Biosci. Bioeng. 5 (2001) 405–416.
[2] F. Ma, M.A. Hanna, Biodiesel production: a review, Bioresour. Technol. 70 (1999) 1–15.
[3] S.V. Ranganathan, S.L. Narasimham, K. Muthukumar, An overview of enzymatic production of biodiesel, Bioresour. Technol. 99 (2008) 3975–3981.
[4] P.V. Lara, E.Y. Park, Potential application of waste activated bleaching earth on the production of fatty acid alkyl esters using Candida cylindracea lipase in organic solvent system, Enzyme Microbiol. Technol. 34 (2004) 270–277.
[5] B. Fu, P.T. Vasudevan, Effect of solvent-co-solvent mixtures on lipase-catalyzed transesterification of canola oil, Energy Fuels 24 (2010) 4646–4651.
[6] L. Li, W. Du, D. Liu, L. Wang, Z. Li, Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium, J. Biomol. Catalys. B: Enzyme 43 (2006) 58–62.
[7] D. Royon, M. Daz, G. Ellenrieder, S. Lotelli, Enzymatic production of biodiesel from cotton seed oil using *t*-butanol as a solvent, Bioresour. Technol. 98 (2007) 648–653.
[8] H.C. Chen, H.Y. Ju, T.T. Wu, Y.C. Liu, C.C. Lee, C. Chang, Y.L. Chung, C.J. Shieh, Continuous production of lipase-catalyzed biodiesel in a packed-bed reactor: optimization and enzyme reuse study, J. Biomed. Biotechnol. (2011) 1–6, Article ID 950725.
[9] M. Iso, B. Chem, M. Eguchi, T. Kudo, S. Shrestha, Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase, J. Mol. Catal. B Enzym. 16 (2001) 53–58.
[10] K.B. Bako, F.C.S. Kova, L. Gubicza, J.K. Hansco, Enzymatic biodiesel production from sunflower oil by *Candida antarctica* lipase in a solvent-free system, Biocatal. Biotransform. 20 (2002) 437–439.
[11] S. Hama, H. Yamaji, T. Fukumizu, T. Numata, S. Tamalampudi, A. Kondo, H. Nodac, H. Fukuda, Biodiesel fuel production in a packed-bed reactor using lipase-producing *Rhizopus oryzae* cells immobilized within biomass support particles, Biochem. Eng. J. 34 (2007) 273–278.
[12] Y. Xu, W. Du, J. Zeng, D. Liu, Conversion of soya bean oil to biodiesel fuel using lipozyme TL IM in a solvent free medium, Biocatal. Biotransform. 22 (2004) 45–48.
[13] A.L. Matassoli, I.N. Correa, M.F. Portilho, C.O. Veloso, M.A. Langone, Enzymatic synthesis of biodiesel via alcoholysis of palm oil, Appl. Biochem. Biotechnol. 155 (2009) 347–355.
[14] W. Du, Y.Y. Xu, J. Zeng, D.H. Liu, Novozym 435-catalysed transesterification of crude soy bean oils for biodiesel production in a solvent-free medium, Biotechnol. Appl. Biochem. 40 (2004) 187–190.
[15] T. Tan, K. Nie, F. Wang, Production of biodiesel by immobilized *Candida sp* lipase at high water content, Appl. Biochem. Biotechnol. 128 (2006) 109–116.
[16] L. Fjerbaek, K.V. Christensen, B. Norddahl, A review of the current state of biodiesel production using enzymatic transesterification, Biotechnol. Bioeng. 102 (2009) 1298–1315.
[17] A.L. Paiva, V.M. Balcao, F.X. Malcata, Kinetics and mechanisms of reactions catalyzed by immobilized lipases, Enzyme Microbiol. Technol. 27 (2000) 187–204.
[18] A. Turkan, S. Kalay, Monitoring lipase-catalyzed methanolysis of sunflower oil by reversed-phase high-performance liquid chromatography: Elucidation of the mechanisms of lipases, J. Chromatogr. A 1127 (2006) 34–44.
[19] V. Dubois, S. Breton, M. Linder, J. Fanni, M. Parmentier, Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential, Eur. J. Lipid Sci. Technol. 109 (2007) 710–732.
[20] C. Plank, J. Lorbeer, Simultaneous determination of glycerol, and mono-, di- and triglycerides in vegetable oil methyl esters by capillary gas chromatography, J. Chromatogr. A 697 (1995) 461–468.
[21] D. Darnoko, M. Cheryan, Kinetics of Palm Oil Transesterification in a Batch Reactor, J. Am. Oil Chem. Soc. 77 (2000) 1263–1267.
[22] B. Freedman, R.O. Butterfield, E.H. Pryde, Variables affecting the yields of fatty esters from transesterified vegetable oils, J. Am. Oil Chem. Soc. 63 (1986) 1375–1380.
[23] P.W. Atkins, J. dePaula, Physical chemistry for the life science, first ed.Oxford Univ Press, Oxford UK, 2006.